Serum antibodies against Panton–Valentine leukocidin in a normal population and during Staphylococcus aureus infection

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Abstract

To determine whether Staphylococcus aureus Panton–Valentine leukocidin (PVL) is expressed during human infection, anti-PVL antibody titres were compared in patients with PVL-positive and PVL-negative staphylococcal infections, and in patients with no evidence of S. aureus infection. Patients with PVL-positive strains had higher levels of anti-PVL antibodies than individuals of both control groups. The median anti-PVL titre increased 8.6-fold during the course of PVL-positive infection and 1.4-fold during PVL-negative infection. These results indicate that only PVL-positive S. aureus strains elicit significant anti-PVL antibody production in humans, and demonstrate the production of PVL during PVL-positive S. aureus infection. The protective role of this immune response remains to be established.

Keywords: Antibody, human, infection, Panton–Valentine leukocidin, Staphylococcus aureus

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Introduction

Staphylococcus aureus is an important human pathogen. It expresses a variety of virulence factors, including Panton–Valentine leukocidin (PVL) [1]. PVL, together with γ-haemolysin and other leukocidins (e.g. LukE, LukD, LukM and LukR), belongs to the family of synergohymenotropic toxins (SHTs), which damage host cell and/or erythrocyte membranes by the synergistic action of two classes of non-associated proteins designated S and F. The F components share 70–80% sequence identity, and the S components 60–80% sequence identity [2]. SHTs are antigenically related [3].

Only PVL has been epidemiologically linked to specific human S. aureus infections, such as primary skin and soft tissue disease, severe necrotizing pneumonia, and severe bone and joint infection [4–6]. These epidemiological links suggest that PVL plays a role in the pathophysiology of these infections. By using isogenic PVL-positive and PVL-negative S. aureus laboratory strains and recombinant toxins in a mouse model of acute pneumonia, it was shown that PVL is sufficient to cause pneumonia [7].

However, several groups recently found no differences in infection severity when isogenic pairs of PVL-positive and PVL-negative clinical strains were used in mouse models [8,9]. It is questionable whether or not PVL is actually produced during human infection. PVL production in vivo could be indirectly demonstrated on the basis of serological changes during infection. Some older publications mentioned anti-PVL antibody production during diseases caused by S. aureus [10–13], but none specified whether the strains produced PVL in vitro. It is therefore difficult to ascertain whether the anti-PVL antibody responses in these patients were caused by PVL itself or by antigenically related SHTs.

The aim of this work was to examine anti-PVL antibody titres in the normal population and in patients with staphylococcal infections due to PVL-negative and PVL-positive strains.

Materials and Methods

Serum specimens

In total, 256 serum samples (from 236 patients) were collected during the year 2002 from three different groups of immunocompetent patients: (i) patients with no evidence of S. aureus infection; (ii) patients with documented PVL-positive S. aureus infections; and (iii) patients with documented PVL-negative S. aureus infections. One hundred and forty-two sera were from 142 patients with no evidence of S. aureus
infection. These patients were divided into 12 age classes as follows: >0 to ≤1 year, >1 to ≤3 years, >3 to ≤6 years, >6 to ≤10 years, >10 to ≤15 years, >15 to ≤20 years, >20 to ≤30 years, >30 to ≤40 years, >40 to ≤50 years, >50 to ≤60 years, >60 to ≤70 years, and >70 years. One hundred and fourteen sera were from S. aureus-infected patients. Forty-three sera were from 31 patients with documented PVL-positive S. aureus infections, comprising ten cases of skin and soft tissue infection, 16 cases of necrotizing pneumonia, and five cases of osteomyelitis. Seventy-one sera were from 63 patients with documented PVL-negative S. aureus infections, comprising 14 cases of skin and soft tissue infection, one case of Kawasaki disease, three cases of pneumonia, 11 cases of bone and/or joint infection, 16 cases of infective endocarditis, and 18 cases of bacteraemia. Sera were stored at −20°C until ELISA testing.

S. aureus was identified on the basis of colony and cell morphology, coagulase testing with rabbit plasma (bio-Mérieux, Marcy l’Etoile, France), and the Staphyslide agglutination test (bioMérieux). Genomic DNA was extracted for PCR analysis with a standard procedure, and its concentration was estimated spectrophotometrically. Sequences specific for the PVL genes (lukS-PV and lukF-PV, forming the luk-PV operon) were detected by PCR as previously described [4].

Human intravenous polyclonal immunoglobulin (IVlg; Tége-line) was purchased from Laboratoire Français du Frac-tionnement et des Biotechnologies (LBF, Courtaboeuf, France). Tége-line is isolated by fractionation of a pool of IgG collected from 20000 healthy donors. It contains >97.6% IgG, composed of IgG1 (58.8%), IgG2 (34.1%), IgG3 (5.4%), and IgG4 (1.7%), IgA (<1.7%), and traces of pepsin. A standard range (50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 mg/L) was prepared from a solution of Tége-line (50 g/L) and used for antibody detection in clinical samples. Tége-line contains PVL-specific antibodies (against LukS-PV and LukF-PV) as shown by ELISA [14].

**Anti-LukS-PV and anti-LukF-PV immunoassays**

Recombinant LukS-PV and LukF-PV (rLukS-PV and rLukF-PV) were purified from Escherichia coli culture supernatants as previously described [15]. Anti-LukS-PV and anti-LukF-PV antibodies were detected in IVlg and sera by using a specific ELISA method with solid-phase rLukS-PV and rLukF-PV and peroxidase-conjugated anti-immunoglobulin antibodies. The procedure was adapted from current protocols in molecular biology [16]. The wells of microtitre plates (Sigma, Saint Quentin Fallavier, France) were coated with 125 mg/L of rLukS-PV and rLukF-PV in phosphate-buffered saline overnight at room temperature, and this was followed by incubation with a blocking solution of phosphate-buffered saline–TWEEN (0.05%) containing lyophilized skimmed milk (10 g/L) for 30 min at 37°C.

Unbound rLukS and rLukF was washed out twice with the blocking solution. Serial dilutions of IVlg (50–0.78 mg/L) (for the standard range) and sera (diluted 1 : 1000–1 : 100 000) were added to duplicate wells for 1 h at 37°C, and then peroxidase-conjugated rabbit anti-human polyclonal IgG (diluted 1 : 30 000) (Sigma) was added after three washes. The microplates were incubated for 1 h at 37°C, and washed before addition of the substrate tetramethylbenzidine (Sigma). The reaction was stopped with 75 μl of 1 M H2SO4.

The plates were read at 450 nm in a Model 680 microplate reader (BioRad, Marnes-la-Coquette, France). The standard curve was linear with an r^2 value of 0.99.

The results were expressed in arbitrary units per litre (AU/L); 1000 arbitrary units correspond to the amount of anti-PVL antibodies contained in a solution of commercial human polyclonal immunoglobulins at 12.5 g/L, which is the physiological human immunoglobulin serum concentration.

**Statistical analysis**

The Mann–Whitney U-test was implemented with SPSS software version 11.0 (SPSS Inc., Chicago, IL, USA) and used to identify differences in anti-PVL titres between groups of sera. The level of statistical significance was set at p < 0.05.

**Results**

**Distribution of PVL antibody levels in patients with no evidence of S. aureus infection**

Anti-PVL antibody levels in individuals free of S. aureus infection ranged from 102 to 15 681 AU/L (median 854 AU/L), and were distributed in log normal mode with an additional small peak at 7000–9000 (Fig. 1). Antibody levels were low in the first three age groups (0–1 year, 1–3 years, and 3–6 years; median 382 AU/L), and significantly increased in the next two age groups of 6–10 years and 10–15 years (median 1502 and 1930 AU/L, respectively; p < 0.001) (Fig. 2). In individuals between 15 and 40 years of age, the anti-PVL level was lower than in the previous two age groups (median 789 AU/L), and then increased slightly with age until 70 years.

**Distribution of PVL antibody levels in infected patients**

Anti-PVL antibody titres in the initial serum samples of patients with PVL-negative S. aureus infection ranged from 40 to 47 645 AU/L (median 1300 AU/L) and were not significantly different from the control values. Titres in patients
with PVL-positive *S. aureus* infection ranged from 200 to 400 000 AU/L (median 10 070 AU/L) and were significantly higher than the titres in patients with no evidence of *S. aureus* infection and patients with PVL-negative *S. aureus* infection (*p* < 0.001) (Fig. 3). For patients with PVL-positive *S. aureus* infection, anti-PVL titres were not significantly different among those with skin and soft tissue infections, necrotizing pneumonia or osteomyelitis.

**Serial samples**
Paired serum samples were available for 12 patients infected with PVL-producing *S. aureus* strains and eight patients infected with PVL-negative *S. aureus* strains. The interval between the two samples ranged from 3 to 210 days, and was not significantly different between the two groups of patients (*p* = 0.70).

The median anti-PVL titre in patients with PVL-producing strains increased from 15 540 in the first sample to 64 953 AU/L in the second sample (range 4609–33 571 and 21 377–125 852 AU/L, respectively) from patients with skin and soft tissue infections, and from 2254 to 46 850 AU/L

**FIG. 1.** Distribution of anti-Panton–Valentine leukocidin (PVL) antibody levels in patients with no evidence of *Staphylococcus aureus* infection.

**FIG. 2.** Distribution of anti-Panton–Valentine leukocidin (PVL) antibody levels in the different age groups of patients with no evidence of *Staphylococcus aureus* infection. Bars show median values.

**FIG. 3.** Distribution of anti-Panton–Valentine leukocidin (PVL) antibody levels in the three populations studied: 142 sera from patients with no evidence of *Staphylococcus aureus* infection (S0); 71 sera from patients with PVL-negative *S. aureus* infection, comprising 63 first sera (S1) and 8 second sera (S2); and 43 sera from patients with PVL-positive *S. aureus* infection, comprising 31 first sera (S1) and 12 second sera (S2). Bars show median values.
(range 387–14 336 and 859–400 000 AU/L, respectively) in the respective samples from patients with necrotizing pneumonia. In patients with PVL-negative S. aureus infection, median anti-PVL titres increased from 2516 to 3302 AU/L (range 1203–3940 and 2198–4187 AU/L, respectively).

The titre in the second sample was almost always higher than that in the first. The ratio of anti-PVL titres of the two samples was significantly higher in patients with PVL-producing S. aureus strains (median 8.6; 4.6 in the case of skin and soft tissue infections and 10.4 in that of necrotizing pneumonia) than in patients with PVL-negative S. aureus strains (median 1.4; p 0.002). The only exception was the single patient who died of necrotizing pneumonia and whose anti-PVL titre fell from 3275 to 859 AU/L.

**Discussion**

The results of this study clearly show that only S. aureus strains harbouring the luk-PV genes elicit significant anti-PVL antibody production in humans. Patients with strains harbouring luk-PV genes had higher levels of anti-PVL antibodies than individuals with no evidence of S. aureus infection. Moreover, the median anti-PVL level increased 8.6-fold during the course of PVL-positive S. aureus infection (12 patients) and only 1.4-fold in patients with PVL-negative S. aureus infection (eight patients). The observed rise in the anti-PVL antibody titre indirectly confirms that PVL is produced during human infection. Direct evidence for this production has been provided by PVL detection in pus from patients with skin infections [17]. Altogether, this suggests that PVL may contribute to the pathophysiology of human S. aureus infection.

Strong responses to PVL were observed in most patients with PVL-positive infections, with three exceptions. In a patient with recurrent furunculosis, the initial anti-PVL antibody titre was already very high (33 571 AU/L), and increased only to 64 953 AU/L; in the only fatal case of necrotizing pneumonia of this survey, the initial anti-PVL titre was 3275 AU/L (i.e. normal for the age), and it fell four-fold 3 days before death. The specific antibodies may have been consumed by PVL in this patient. In another case of necrotizing pneumonia, the patient relapsed 20 days after the first episode. The anti-PVL antibody titre increased only from 520 to 981 AU/L during this period. As anti-PVL antibodies can neutralize PVL activity [14], it is tempting to speculate that the weak immune response to PVL in these two patients could have negatively affected their outcome. A significant increase in the anti-PVL titre may favourably influence clinical outcome [10,13,18]. Johanovsky [18] observed a negative correlation between the severity of human S. aureus infection and the anti-leukocidin titre. Panton and Valentine also suggested in 1932 that ‘anti-leukocidin antisera or vaccine could be a useful therapeutic approach for serious PVL-producing S. aureus strains infections which did not respond well to available treatment’ [10].

In 1965, Mudd et al. [19] tested PVL toxoid injection as an adjunctive treatment for disease caused by S. aureus, and observed an increase in anti-PVL titres and a beneficial effect in patients with osteomyelitis and soft tissue infection.

Another question concerns host susceptibility to PVL and the possible link between absence of protective antibodies and outcome of PVL-positive infection. However, in this study, patients with PVL-positive infection had higher anti-PVL antibody titres than patients with PVL-negative infection. As anti-PVL antibodies neutralize PVL activity, and most of the patients developed strong immune responses to PVL, we suspect that the immune response was already underway when the first samples were obtained, making it impossible for us to address this question.

In subjects with no evidence of S. aureus infection, there was a particular age distribution of anti-PVL titres. As expected, levels of anti-PVL antibodies increased gradually with age, but a peak between 6 and 15 years was observed. No such peak has been observed with other S. aureus antigens, e.g. whole bacteria, cell wall teichoic acid, peptidoglycan, and exfoliative toxins. Anti-S. aureus antibody titres usually increase continuously until adolescence, and then level off [20–23]. It will be interesting to confirm this particular age distribution of anti-PVL in a larger population and to determine whether it correlates with age of colonization or infection by PVL-positive S. aureus.

In conclusion, the results of this study clearly show that only strains harbouring luk-PV genes can elicit significant anti-PVL antibody production in humans, indirectly confirming PVL production during human infection. The protective role of this immune response remains to be determined.

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**Transparency Declaration**

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References